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Phylogenetic Relationships of the *Wolbachia* of Nematodes and Arthropods

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***Wolbachia* are well known as bacterial symbionts of arthropods, where they are reproductive parasites, but have also been described from nematode hosts, where the symbiotic interaction has features of mutualism. The majority of arthropod *Wolbachia* belong to clades A and B, while nematode *Wolbachia* mostly belong to clades C and D, but these relationships have been based on analysis of a small number of genes. To investigate the evolution and relationships of *Wolbachia* symbionts we have sequenced over 70 kb of the genome of wOvo, a *Wolbachia* from the human-parasitic nematode *Onchocerca volvulus*, and compared the genes identified to orthologues in other sequenced *Wolbachia* genomes. In comparisons of conserved local synteny, we find that wBm, from the nematode *Brugia malayi*, and wMel, from *Drosophila melanogaster*, are more similar to each other than either is to wOvo. Phylogenetic analysis of the protein-coding and ribosomal RNA genes on the sequenced fragments supports reciprocal monophyly of nematode and arthropod *Wolbachia*. The nematode *Wolbachia* did not arise from within the A clade of arthropod *Wolbachia*, and the root of the *Wolbachia* clade lies between the nematode and arthropod symbionts. Using the wOvo sequence, we identified a lateral transfer event whereby segments of the *Wolbachia* genome were inserted into the *Onchocerca* nuclear genome. This event predated the separation of the human parasite *O. volvulus* from its cattle-parasitic sister species, *O. ochengi*. The long association between filarial nematodes and *Wolbachia* symbionts may permit more frequent genetic exchange between their genomes.**

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Introduction

Wolbachia are alphaproteobacteria that live intracellularly in a range of animal hosts [1]. *Wolbachia* belong to the Anaplasmataceae in the Rickettsiales, a diverse group of intracellular symbionts. In other Rickettsiales, the symbiosis is usually parasitic or pathogenic, and many of these bacteria cause significant human and veterinary disease problems. Rickettsiales have also been identified as symbionts of arthropods, and are implicated in causing reproductive manipulations in their hosts similar to those of *Wolbachia*. (See below; we note that our knowledge of these bacteria is likely to have a severe ascertainment bias, as disease-causing pathogens are obvious and important, whereas innocuous or even beneficial interactors, and free-living species, will be missed. In this context it is informative that unbiased surveys of ecosystems using PCR amplification of conserved genes are turning up rickettsia-like bacteria in many unexpected situations [2].)

In arthropods, where they were first discovered, *Wolbachia* are the causative agents of a number of fascinating reproductive manipulations [3]. These manipulations serve to promote the survival of infected female arthropods, which pass the *Wolbachia* vertically to their offspring. A range of phenotypes are caused by *Wolbachia* infection in arthropods, including killing or feminisation of genetic males, induction of parthenogenetic reproduction in haplo-diploid females, and induction of reproductive incompatibility between individuals that do not have the same infection status. The prevalence of *Wolbachia* in current arthropod faunas is very high [4,5]; this is due to rare but successful horizontal transfer of the infection between taxa, and is likely to play a role in

speciation. Selective sweeps caused by introgression of new *Wolbachia* strains have strongly shaped mitochondrial population genetics [6], and genomic conflict between the bacterium and the nuclear genome may promote reproductive isolation [7]. There is limited congruence between host and bacterial phylogenies in the arthropod system.

Most arthropod *Wolbachia* derive from two relatively closely related clades, called A and B [1]. The only formally named *Wolbachia* is *W. pipiens* from the mosquito *Culex pipiens*, but divergence between the major clades is similar to that observed between species in other bacterial genera [8]. Variant arthropod *Wolbachia* have been described, from springtails, termites, and spiders, that define additional, more deeply separated clades (E, F, and G) [8,9]. Resolution of the relationships of these additional clades is currently poor. However, *Wolbachia* “infections” are not limited to the

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Abbreviations: GC%, proportion of the sequenced segments made up of guanine and cytosine bases; idel, insertion/deletion; ML, maximum likelihood; NJ, neighbour joining; SH, Shimodaira-Hasegawa; wAna, *Wolbachia* from *Drosophila ananassae*; wBm, *Wolbachia* from *Brugia malayi*; wMel, *Wolbachia* from *Drosophila melanogaster*; wOoc, *Wolbachia* from *Onchocerca ochengi*; wOvo, *Wolbachia* from *Onchocerca volvulus*; wSim, *Wolbachia* from *Drosophila simulans*

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Synopsis

Filarial nematode worms cause hundreds of millions of cases of disease in humans worldwide. As part of efforts to identify new drug targets in these parasites, the Filarial Genome Project rediscovered that these worms carry within them a symbiotic bacterium, which may be a novel target. Fenn et al. investigated the relationships of these bacteria, from the genus *Wolbachia*, to those previously identified in arthropods using a new dataset of genome sequence data from the human parasite *Onchocerca volvulus*. *O. volvulus* causes river blindness in West Africa. The authors found that the *Wolbachia* strains found in nematodes are more closely related to each other than they are to the *Wolbachia* in insects, suggesting that the nematodes and their bacterial partners have been coevolving for some considerable evolutionary time and may indeed be good targets. In addition, the authors identified a fragment of *Wolbachia* DNA that was inserted in the genome of its nematode host and has subsequently degenerated. The insertion occurred before *O. volvulus* diverged from another nematode species, *O. ochengi*, found in cattle.

Arthropoda. Parasitic filarial nematodes of the Onchocercidae, including several major human pathogens, harbour intracellular *Wolbachia* [10–12]. No other nematodes are currently known to harbour *Wolbachia* [13], though other nematode–bacterial symbioses are common. In the onchocercids, the *Wolbachia* can be divided into two major clades, C and D [14], which, unlike the arthropod *Wolbachia* clades, show phylogenetic congruence with their hosts [15]. Thus, closely related filarial nematodes have closely related *Wolbachia*, and the association between nematode and bacterium appears to be one of long-term (>100 million years), stable, vertical transmission. The *Wolbachia* of one filarial species, *Mansonella ozzardi*, has been placed by analysis of a small number of genes in clade F with termite and weevil isolates.

Analysis of the relationship between the nematodes and their symbionts has revealed that they are likely to be mutualists [16]. Killing the bacteria with tetracycline affects nematode growth, moulting, fecundity, and lifespan [17,18]. In arthropods, in most cases, tetracycline treatment yields cured, healthy hosts, and related parasitic nematodes that do not harbour *Wolbachia* are unaffected by tetracycline treatment [18]. This feature of nematode–*Wolbachia* interaction has led to trialling of tetracycline antibiotics for treatment of human filariases, with very positive results [19–22].

In the Rickettsiales and *Wolbachia*, therefore, where the intracellular habit is ancestral, there has either been a loss of the parasitic or pathogenic phenotype in the nematode *Wolbachia* or evolution of novel parasitic mechanisms in the arthropod *Wolbachia*. Previous analyses of *Wolbachia* phylogeny, and of the relationships of the genus to other Rickettsiales, have been based on very few genes (the *Wolbachia* surface protein *wsp*, cell-division protein *ftsZ*, citrate synthase *gltA*, *groEL* chaperone, and small subunit ribosomal RNA [16S] genes) [1,14,15,23]. These analyses were equivocal concerning the deeper structure of the *Wolbachia*, and could not resolve the placement of the root of the genus; clades E, F, and G are significantly under-sampled. A major limiting factor has been the inferred length of the branches leading to the outgroup taxa. As the genes sequenced have generally been chosen for their ability to resolve within-clade, between-isolate relationships, they are not suited to robust

resolution of the deeper relationships of *Wolbachia*. Studies on yeasts and other taxa have shown that extended, multigene datasets can often provide robust resolution when individual constituent genes cannot [24].

Given that clades A and B are very closely related, two possibilities seem most likely. The first is that the nematode symbionts and the arthropod parasites form two distinct radiations (i.e., the tree has the form [outgroup[[A,B],[C,D]]]; Tree 1 of Figure 1). The second is that one of the nematode symbiont clades (most probably clade C, found in *Onchocerca* species and close relatives) arises basal to the other clades (i.e., the tree has the form [outgroup[C[D[A,B]]]; Tree 2). A final possibility is that nematode *Wolbachia* arose from within the arthropod-infecting clades (Tree 4). Trees 1 and 4 have been implicit in many discussions of *Wolbachia* evolution, possibly because of the historical accident that arthropod *Wolbachia* were the first to be identified, and are the more widely studied. We have generated genome sequence from a clade C *Wolbachia*, *wOvo* from the human parasite *Onchocerca volvulus*, and here analyse it along with genome sequence from the *Wolbachia* of *Drosophila melanogaster* (*wMel*) (clade A), *Wolbachia* from *Brugia malayi* (*wBm*) (nematode, clade D), and a series of anaplasmatacean outgroups to re-examine this question. We find that the root of *Wolbachia* is robustly placed between clades A and [C and D], and thus that the mutualist nematode symbionts likely arose from parasitic or pathogenic ancestors. The close coevolution of nematodes and their *Wolbachia* is underlined by the discovery of a segment of the *Wolbachia* genome translocated to the *O. volvulus* nuclear genome.

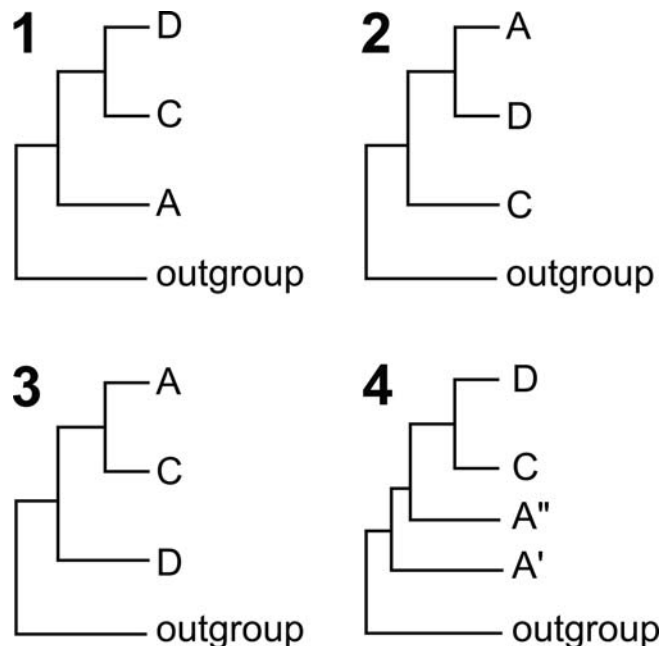


Figure 1. Hypotheses of *Wolbachia* Relationships

While we analysed seven taxa, they can be treated as if they were four: outgroups (*Anaplasma* and *Ehrlichia*), clade A *Wolbachia* (*wMel*, *wAna*, and *wSim*), clade D *Wolbachia* (*wBm*), and clade C *Wolbachia* (*wOvo*). There are thus three possible placements of the root of *Wolbachia*: (1) Tree 1 [outgroups[A(C,D)]]; (2) Tree 2 [outgroups[C(D,A)]]; and (3) Tree 3 [outgroups[D(C,A)]]; As clade A included more than one taxon, trees with clade A paraphyletic are also possible. In practice only one such arrangement was found (Tree 4; [outgroups[A' A''(C,D)]]), and may have arisen from analysis of paralogous genes.

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Table 1. PCR Primers Used

Primer Use	Primer Name	Primer Sequence
Primers used to generate probes	Ov_wsp_F1	TTTTATGGCTGGTGGTAGTGC
	Ov_wsp_R1	TGCTAGAGGATTGCTGAGATATGC
	AI111287_F1	TGCTGGAAACGACAATAACC
	AI111287_R1	CTGCTGGCAGGAGTTAGC
	AI261163_F1	CACAGGACTCTGCAACACG
	AI261163_R1	TCAAAGCCTCCACCTATCC
	AW330455_F1	GCGTGGAGGTCTAAAGTTGC
	AW330455_R1	CAACACCACCTGATAATTTTGC
	AW351423_F1	GGGTAAAAGCAAACCTCACTCG
	AW351423_R1	TTTAGGATAAGTGCGAGCATTC
Lambda vector primers for long-range PCR and sequencing	L_FIX_EXP_F1	GAGCTCTAATACGACTCACTATAGGGCGT
	L_FIX_EXP_R1	CTCACTAAAGGGAGTCGACTCGAGC
	Lambda_FIX_T3seq2	CACTAAAGGGAGTCGACTCG
	pBACe3.6_T7	TAATACGACTCACTATAGGG
Primers for PCR and sequencing of <i>wOvo HtrA</i>	wOvo_HtrA_F1	CGTACTTTACCAAATAATAG
	wOvo_HtrA_F2	GTAACACTCGGTAATTCTG
	wOvo_HtrA_R1	GCTAAAGTGTTATAGCGGC
Primers used to identify the <i>Wolbachia</i> nuclear insert in <i>Onchocerca</i> sp.	TATA_F	GTTAAATGTCATTCTAATGA
	TATA_R	TTTCTGGACCTACCGAA
	TATA_Phos	TTGCTGGYRARTTAAGCG
	TATA_OW4C	CCTACYAGTAAAYKAGAT
	Phos	TTGTGTGTTGATTCTTAAG
	OW4C	CTCCAATTCACATGGT

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Results

Five Segments of the Genome of *Wolbachia* from *O. volvulus*

Twenty-seven primer pairs derived from a range of putative genes from *Wolbachia* from *O. volvulus* (*wOvo*) were tested and yielded 11 probes (Table 1). Five of these identified positive clones in the *O. volvulus* genomic libraries, and the inserts of these clones were amplified by long-range PCR and sequenced (Table 2). The total unique sequence length of the segments is 70,830 bp, representing 6.5% of the estimated 1.1 Mb of the *wOvo* genome [25]. The proportion of the sequenced segments made up of guanine and cytosine bases (GC%) ranged from 31.8% to 35.38% with a mean value of 32.9%). The average GC% of *wBm*, *wMel*, and *Rickettsia prowazekii* is 34%, 35.2%, and 29.1%, respectively.

We identified 51 protein-coding genes and three ribosomal RNA genes (16S, 23S, and 5S) in the five segments (Table 3; Figure 2). Coding regions cover 76.6% of the total sequence, again within the expected range when compared to *wBm*,

wMel, and *R. prowazekii* (67.4%, 81%, and 76%, respectively). This corresponds to a gene density of 0.72 protein-coding genes/kb, which is comparable to *wBm* and *R. prowazekii* (both 0.75 functional genes/kb) but much less than *wMel* (0.94 functional genes/kb). If the genome of *wOvo* is similar in size to those of *wMel* and *wBm*, it is estimated to have approximately 800 functional genes, like *wBm* (which has 806) [26], but many fewer than *wMel* (1,270) [27].

Functional annotation was possible for the majority of the 51 protein-coding genes [26,27] (Table 3). Six are *Wolbachia*-specific, having no orthologue in any of the alphaproteobacterial genomes examined, or elsewhere. These include *Wolbachia* surface protein and five conserved hypothetical proteins. As these genes are present only in *Wolbachia*, they may encode proteins involved in the particular symbiotic biology of the bacteria. One gene, OW2-I, is *wOvo*-specific: no function can be ascribed by similarity. A partial pseudogene similar to an ATP-dependent caseinolytic protease ATP-binding subunit, *ClpA*, was identified (Figure 3). An orthologous *ClpA* gene is intact in *wMel* [27], is degraded in *wBm* [26], and is missing from *R. prowazekii* [28]. While it is possible that there is another copy of *ClpA* in the *wOvo* genome, this seems unlikely given the synteny of *wOvo ClpA* and flanking genes with *wMel* and *wBm* (see below). *wBm ClpA* is intact at the 3'/C-terminal end, but has a deletion of 21 bases and two in-frame stop codons compared to *wMel ClpA*. In the region that overlaps with the partial *wOvo ClpA*, the *wBm* representative is intact, while *wOvo* has 13 insertion/deletion (indel) changes, 12 of which cause frame shifts (Figure 3). *ClpA* acts as a molecular chaperone, and when in complex with the protease *ClpP* (*ClpAP*) it recognises and targets proteins for degradation. *ClpX*, another *Clp* regulator, is distinct from *ClpA*, and also forms complexes with *ClpP* (*ClpXP*) [29]. Both *ClpP* and *ClpX* are present in the genomes of *wBm*, *wMel*, and

Table 2. Genome Sequence from *wOvo*

Fragment Name	Length (bp)	%GC	Number of Protein-Coding Genes	Other Genes Identified
OW1	12,024	32.16	11	
OW2	11,498	31.80	10	16S rRNA
OW3	15,081	35.38	8	23S and 5S rRNA
OW4	17,997	32.07	12	
OW5	14,230	32.91	10	1 pseudogene

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Table 3. Genes Identified in Genomic Sequence from wOvo, and Their Support for Different Phylogenies

wOvo Gene on Fragment ^a Name	Co-Ordinates	Gene Annotation	Comparator Taxa ^b		Neighbour Joining		MrBayes		Maximum Likelihood		Alignment Length ^c				
			wMel	wAna	wSim	wBm	E. rum.	E. can.	A. mar.	Tree Supported ^d (%)		Posterior Probability (%)	Preferred Tree ^d	SH Test p-Value	
OW1_A_838-2223		Amidophosphoribosyltransferase	Y	Y	Y	Y	Y	Y	Y	1	100	1	1	0.071	475
OW1_B_2658-3242		NifU-related protein	Y	Y	Y	Y	Y	Y	Y	2	79	1	1	0.377	195
OW1_C_3197-4480		Histidyl-tRNA synthetase	Y	Y	Y	Y	Y	Y	Y	2	80	1	1	0.172	443
OW1_D_4497-4994c		Hypothetical protein	Y	Y	.	Y	.	Y	Y	2	100	1	1	0.453	178
OW1_E_4987-5880c		RNA polymerase sigma-32 factor	Y	Y	.	Y	Y	Y	Y	2	100	2	2	0.225	322
OW1_F_6163-6888c		Wolbachia surface protein	Y	Y	.	Y	.	.	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e	
OW1_G_7185-7517c		Conserved hypothetical protein	Y	Y	.	Y	— ^f	— ^f	3	80	100	3	100	— ^f	110
OW1_H_7521-9005c		DnaX/DNA polymerase III tau subunit	Y	Y	Y	Y	Y	Y	2	100	100	2	2	0.144	542
OW1_I_9370-10023		Glutathione S-transferase	Y	.	Y	Y	Y	Y	1	92	100	1	1	0.117	242
OW1_J_10025-10675c		O-methyltransferase	Y	Y	Y	Y	Y	Y	2	93	1	1	1	0.183	221
OW1_K_11229-11924c		Hypothetical protein	Y	.	Y	Y	Y	Y	2	73	4	1	1	0.263	274
OW2_A_92-451		5' truncated Smr family protein	Y	Y	.	Y	Y	Y	2	97	1	56	1	0.18	245
OW2_B_849-1457c		Hypothetical protein	Y	Y	Y	Y	Y	Y	.	1	100	1	1	0.217	221
OW2_C_165-1575-3088		16S ribosomal RNA gene	Y	Y	Y	Y	Y	Y	2	72	100	2	1	0.181	1,766
OW2_D_3231-3608		Succinate dehydrogenase b-type cytochrome subunit (SdhC)	Y	Y	.	Y	Y	Y	1	59	92	1	1	0.092	136
OW2_E_3605-3970		Succinate dehydrogenase hydrophobic membrane anchor (SdhD)	Y	Y	Y	Y	Y	Y	2	82	99	1	1	0.411	126
OW2_F_4056-5303		Wolbachia-specific hypothetical protein	Y	Y	Y	.	.	.	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e	
OW2_G_6430-7659		Hypothetical protein	Y	Y	Y	Y	Y	Y	2	74	2	55	1	0.417	414
OW2_H_7601-8383		Hypothetical protein	Y	.	Y	Y	Y	Y	2	94	1	99	1	0.399	281
OW2_I_8338-9600		Hypothetical protein	Y	Y	Y	Y	Y	Y	2	98	2	100	2	0.166	418
OW2_J_9855-10652c		wOvo-specific hypothetical protein	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e	
OW2_K_10465-11496c		5' truncated phosphomannomutase	Y	Y	Y	.	Y	Y	2	56	U	2	2	0.489	491
OW2_L_235855-70-3216c		23S and 5S ribosomal RNA gene	Y	Y	Y	Y	Y	Y	2	66	2	83	1	0.325	3,208
OW2_M_3381-4454		Hypothetical protein	Y	Y	Y	Y	Y	Y	1	68	1	100	1	0.082	415
OW2_N_4464-6293		Ribonucleoside-diphosphate reductase alpha chain	Y	Y	Y	Y	Y	Y	U	1	97	1	1	0.158	610
OW2_O_6351-6761		Holliday junction resolvase	Y	Y	.	Y	Y	Y	1	60	1	63	1	0.375	164
OW2_P_7080-8732c		Excinuclease ABC subunit	Y	Y	Y	Y	.	Y	2	92	U	1	1	0.439	621
OW2_Q_8729-10834c		Glycyl-tRNA synthetase, beta chain	Y	Y	Y	Y	Y	Y	2	96	1	100	1	0.076	718
OW2_R_10838-11677c		Glycyl-tRNA synthetase, alpha chain	Y	Y	Y	Y	Y	Y	2	97	2	88	1	0.326	283
OW2_S_11735-12481c		Hypothetical protein	Y	Y	.	Y	Y	Y	2	84	100	3	1	0.308	262
OW2_T_12629-15067c		DNA mismatch repair protein (mutS)	Y	Y	Y	Y	Y	Y	2	100	2	100	2	0.249	862
OW2_U_1359-2072		Hypothetical protein	Y	Y	Y	Y	Y	Y	2	50	100	2	2	0.107	277
OW2_V_2072-2851		Exodeoxyribonuclease III	Y	.	Y	Y	Y	Y	2	93	98	2	2	0.354	281
OW2_W_3167-4564		Wolbachia-specific hypothetical protein	Y	.	Y	.	.	.	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e	
OW2_X_5062-7269		Wolbachia-specific hypothetical protein	Y	Y	Y	.	.	.	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e	
OW2_Y_9216-10694c		Serine protease (HtrA)	Y	Y	Y	Y	Y	Y	2	100	100	2	2	0.108	513
OW2_Z_10698-11570c		Protease subunit (HtrC)	Y	.	Y	Y	Y	Y	2	98	100	2	2	0.156	293



Table 3. Continued

wOvo Gene on Fragment ^a Name	Co-Ordinates	Gene Annotation	Comparator Taxa ^b			Neighbour Joining			MrBayes		Maximum Likelihood		Alignment Length ^c		
			wMel	wAna	wSim	wBm	E. rum.	E. can.	A. mar.	Tree Supported ^d (%)	Bootstrap Support (%)	Tree Supported ^d (%)		Posterior Probability (%)	SH Test p-Value
OW4_G 11573–12607c		Protease subunit (HflK)	Y	.	Y	Y	Y	Y	Y	2	100	1	0.396	371	— ^e
OW4_H 12625–12837c		<i>Wolbachia</i> -specific hypothetical protein	Y	.	Y	Y	.	.	— ^e	— ^e	— ^e	— ^e	— ^e		
OW4_I 13026–14345		<i>Wolbachia</i> -specific hypothetical protein	Y	.	.	Y	.	.	— ^e	— ^e	— ^e	— ^e	— ^e		
OW4_J 14649–14966		Divalent cation tolerance protein	Y	Y	.	Y	Y	Y	98	2	100	2	0.182	127	
OW4_K 15067–15618		Hypothetical protein	Y	Y	Y	Y	Y	.	81	3	68	2	0.213	211	
OW4_L 16658–17956c		Heat shock protein (GroEL)	Y	Y	Y	Y	Y	Y	U	1	100	1	0.079	555	
OW5_A 1204–2712		NADH-ubiquinone oxidoreductase subunit	Y	Y	Y	Y	Y	Y	Y	1	89	1	0.083	523	
OW5_B 2745–3650c		Hypothetical protein	Y	Y	Y	Y	Y	Y	98	1	69	1	0.325	337	
OW5_C 3643–4374c		Geranyltransferase	Y	Y	Y	Y	Y	Y	78	1	70	1	0.216	364	
OW5_D 4526–4798		Acyl carrier protein	Y	Y	Y	Y	— ^f	— ^f	84	U	— ^f	— ^f	— ^f	95	
OW5_E 4807–6060		3-oxoacyl-synthase II	Y	Y	Y	Y	Y	Y	56	1	99	1	0.134	433	
OW5_F 6368–7297		Conserved hypothetical protein	Y	Y	Y	Y	Y	Y	87	3	97	1	0.273	317	
OW5_G 7360–8835c		Glutaryl-tRNA amidotransferase subunit A	Y	Y	Y	Y	Y	Y	79	2	100	2	0.286	495	
OW5_H 9537–10268c		Ribonuclease III	Y	Y	.	Y	Y	Y	65	1	99	2	0.376	246	
OW5_I 10326–11423c		Dihydroorotate dehydrogenase	Y	Y	Y	Y	Y	Y	81	1	54	1	0.266	390	
OW5_J 12317–13213c		Fructose-bisphosphate aldolase	Y	Y	Y	Y	Y	Y	60	2	92	2	0.227	307	
OW5_K 13339–13536c		5' truncated ClpA pseudogene	Y	Y	Y	Y	— ^g	Y	— ^g	— ^g	— ^g	— ^g	— ^g		

^a“c” indicates a gene on the complementary strand.^bA. mar., A. marginale; E. can., E. canis; E. rum., E. ruminantium.^cThe alignment length is in amino acids for protein-coding genes and in bases for the ribosomal RNA genes.^dTrees supported as illustrated in Figure 1. Entries in red have significant support (i.e., >70% bootstrap support under NJ and >90% posterior probability under Bayesian analysis).^eNo analysis was possible because the gene is specific to *Wolbachia*.^fFor OW1-G and OW5-D, *R. prowazekii* and *R. typhi* orthologues were used as outgroups.^gNot analysed because wBm orthologue is a pseudogene.

U, unresolved polytomy.

DOI: 10.1371/journal.ppat.0020094.t003

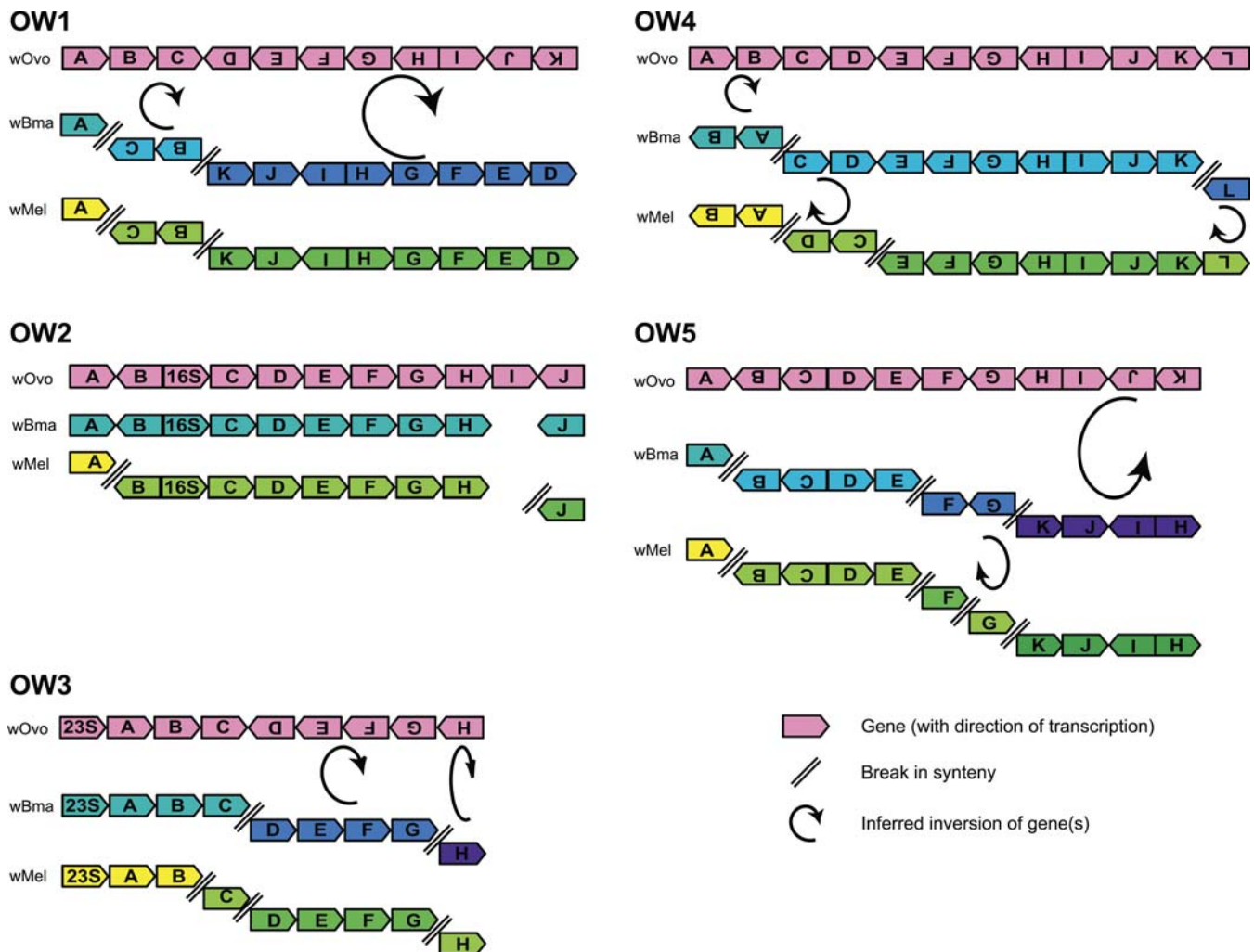


Figure 2. Synteny Comparisons between *wOvo*, *wMel*, and *wBm*

Cartoons represent the conservation of local synteny between the sequenced *wOvo* fragments (OW1 to OW5) and the orthologous regions of the genomes of *wBm* and *wMel*. Genes are shown by small arrowed boxes, but are not drawn to scale. Double diagonal lines show breaks in synteny, and curled arrows show inversions of orientation.

DOI: 10.1371/journal.ppat.0020094.g002

R. prowazekii. It has been reported that *ClpAP* and *ClpXP* have distinct substrate specificities in that *ClpXP* binds only substrate proteins that contain a recognition signal [30]. The benefits for mutualist *Wolbachia* of not having *ClpA* are unclear, as *ClpA* is the more generalist subunit, dealing with proteins damaged by heat shock and starvation.

A second *wOvo* serine protease subunit, identified as *HtrA*, was found in fragment OW4 (gene OW4-E). A *HtrA* from *wOvo* has been reported previously [31], but OW4-E differs from the published sequence, particularly in the 3' half of the gene. Resequencing of *wOvo* *HtrA* from *O. volvulus* genomic DNA yielded the same sequence as OW4-E. No fragments or sequences corresponding to the published *HtrA* were recovered. Alignment of OW4-E and other alphaproteobacterial *HtrA* genes and the published sequence revealed many single base changes and several indel events that change the frame of the translated protein with respect to other *HtrA* sequences. The 3' end of the published "*wOvo*" *HtrA* is, however, identical to *wBm* *HtrA*, while the 5' end is nearly identical to OW4-E: it is likely to be an artefactual fusion

between *wOvo* and *wBm* genes, with some indel sequencing errors also.

Synteny Comparisons between *wOvo*, *wBm*, and *wMel*

The arrangement of genes in the five fragments of the *wOvo* genome was compared to the sequenced genomes of other *Wolbachia* and Anaplasmataceae. None of the five *wOvo* fragments was fully syntenic with either fully sequenced *Wolbachia* (Figure 2). Fragment OW2 differed from *wBm* only in the presence of a *wOvo*-specific coding sequence (OW2-I). The other *wOvo* fragments had two or three rearrangements compared to *wBm*. Comparison to *wMel* identified between two and four rearrangements per fragment. Overall, *wMel* and *wBm* were more similar to each other in the compared regions, sharing many gene order structures compared to *wOvo*. Of the five instances where rearrangements compared to *wOvo* differ between *wMel* and *wBm*, *wBm* is more like *wOvo* in four (Figure 2). In the fifth (in OW4), gene OW4-L is inverted, but still linked, in *wMel*, while it is unlinked (but in the same transcriptional orientation) in *wBm*. None of the gene arrangements specific to *wOvo*, *wBm*, or *wMel* were



Figure 3. A *ClpA* Pseudogene in *wOvo* Has Many Inactivating Mutations

An alignment of the nucleotide sequences of *ClpA* from *wMel* (a functional gene) and *wBm* (a pseudogene, inactivated by mutations that generate two in-frame stop codons; otherwise intact), and the partial gene identified from *wOvo* from fragment OW5. The *wOvo* gene has multiple, independent, inactivating mutations in the 5' region available for comparison. Yellow shading indicates in-frame indel events, red shading indicates frame-shifting indel events (observed only in *wOvo* OW5-K), and violet shading indicates the two in-frame stop codons in *wBm ClpA*.

DOI: 10.1371/journal.ppat.0020094.g003

found in the other Anaplasmataceae genomes surveyed (unpublished data).

Phylogenetic Analyses of *Wolbachia* Based on 46 Genes

We identified putative orthologues for the genes identified on the *wOvo* fragments from the complete and partial genomes of *wBm*, *wMel*, *Wolbachia* from *D. ananassae* (*wAna*), *Wolbachia* from *D. simulans* (*wSim*), *Ehrlichia canis*, *E. ruminantium*, and *Anaplasma marginale*. For each gene, we collected all homologues from all sequenced genes from alphaproteobacteria, constructed alignments, and analysed these phylogenetically using the neighbour joining (NJ) algorithm. For the set of target taxa (see Table 3) we selected those homologues that were robustly defined as orthologous to the *wOvo* genes.

For two proteins (OW1-G and OW5-D) no orthologues were identified in *Ehrlichia* or *Anaplasma*, and for these we selected orthologues from *R. typhi* and *R. prowazekii* as outgroups. Calculation of the distance from each *wOvo* protein to that of *E. canis*, compared to its *wMel* or *wBm* orthologue, showed that there was no obvious long branch artefact that might artificially associate two of the three *Wolbachia*, and that the set of genes analysed embody a wide range of evolutionary rates (Figure 4). The gene set is thus suited to analysis of both local and deep phylogenetic problems [24].

Each alignment of orthologues was then subjected to phylogenetic analysis using NJ, maximum likelihood (ML), and Bayesian ML models. The use of multiple methods of analysis is of utility in the identification of sequences or

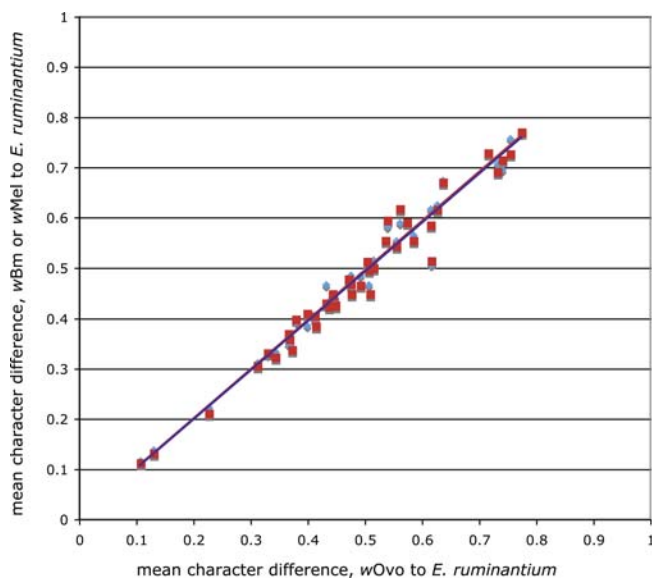


Figure 4. The wOvo Genome Has Similar Evolutionary Rates to wMel and wBm

The graph shows the relationship between evolutionary rates (mean difference) for all 46 protein-coding genes, calculated as distance to the outgroup *E. ruminantium*, between wOvo and wMel (red) and wOvo and wBm (blue). For both comparisons, the slope of the line is $\cong 1$ (wOvo/wMel 0.977 ± 0.002 ; wOvo/wBm 0.981 ± 0.002), indicating that while wOvo has a lower rate than that of the other *Wolbachia* the difference is minor ($\sim 2\%$ overall).

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clades that behave differently or aberrantly under one method compared to others. The Bayesian ML analytical method is generally recognised to be very effective in dealing with biases in sequence alignments, though it is not foolproof [32]. NJ, as it effectively reduces all signal to a single pairwise difference, is most liable to systematic error. Under NJ, 28 of the 44 protein-coding genes yielded support (bootstrap values $> 70\%$) for a close relationship between wMel and wBm to the exclusion of wOvo (i.e., Tree 2 of Figure 1; Table 4). Two genes supported Tree 1 and one Tree 3; the other genes did not yield phylogenies with $>70\%$ bootstrap support for any of the trees. Under Bayesian ML, only 15 of the individual proteins supported Tree 2 with significant posterior probability ($>90\%$), while 11 supported Tree 1. Tree 3 was supported under Bayesian analysis of the same protein, OW1-G, that yielded Tree 3 in the NJ analysis. We note that we had to use *Rickettsia* outgroups for this gene as no orthologues were identified in *Ehrlichia* or *Anaplasma*

genomes, and that this may have resulted in a long branch artefact. The ribosomal RNA genes yielded support for Tree 2 in NJ and Bayesian ML analyses, though the support was low. Surprisingly, despite the strong support for distinct trees by both methods for many genes, Shimodaira-Hasegawa (SH) tests found no cases in which there was a significant difference in support for Trees 1 or 2 (Table 3).

Bayesian ML analyses were also carried out on a concatenated alignment of 42 protein-coding genes (excluding those lacking *Anaplasma* and *Ehrlichia* outgroups) using two models of protein evolution. The first used a single rate for all the sequences, while the second, more realistic model allowed each protein to evolve with its own rate multiplier. The second model was significantly better (harmonic mean LnL partitioned = $-121,745.01$; unpartitioned = $-122,039.86$; Bayes factor $\cong e^{294} \cong 10^{127}$). Using a single rate yielded Tree 1, a result that might be expected considering the relative lengths of the proteins supporting Tree 1 versus Tree 2 (Table 3). A SH test showed highly significant support for Tree 1 ($p = 0.003$). Analysis using the partitioned model yielded Tree 1 with high posterior probabilities at all nodes (Figure 4). Although Bayesian ML analysis can overestimate support for trees, this result was found in multiple independent analyses.

Identification of a Lateral Gene Transfer Event from *Wolbachia* to the Nematode Nuclear Genome

Comparison of the sequenced wOvo genomic fragments to available *O. volvulus* DNA sequences identified a segment of *O. volvulus* genomic DNA that had significant nucleotide sequence identity to two distinct genes in wOvo (Figure 5). A 5,074-bp EcoRI fragment of *O. volvulus* genomic DNA had been isolated and sequenced because it contained a TATA box-binding protein gene (GenBank accession L13731) [33]. The TATA box-binding protein gene is located from residues ~ 2200 to 3500 of the fragment, but a full-length coding sequence was not predicted previously [33]. We resurveyed this sequence, identifying a likely 5' *trans*-splice acceptor site at bases 2096 to 2101 and an initiation ATG at 2105 to 2107. The ~ 2 kb upstream of this *trans*-splice acceptor site are free of obvious coding features and have no BLASTx matches in public databases (unpublished data). We identified a region of 104 bases (from position 182 to 384 of L13731) that was 63% identical to wOvo OW4-C (Figure 6). There are three insertions (totalling four bases) and one deletion (of one base) in L13731 compared to wOvo OW4-C. Immediately following this section in L13731 is a stretch of 205 bases (385 to 589) that is 84% identical to wOvo OWJ-2 (with two insertions, of one base and 13 bases, and one deletion of one

Table 4. Summary of Support under Different Models of Phylogenetic Inference

Mode of Inference	Number of Genes Supporting Tree 1	Number of Genes Supporting Tree 2	Number of Genes Supporting Tree 3
NJ ^a	8 (1 with support $> 90\%$)	32 (17 with support $> 90\%$)	3 (0 with support $> 90\%$)
MrBayes ^b	19 (10 with support $> 95\%$)	19 (14 with support $> 95\%$)	4 (3 with support $> 95\%$)
ML	30	14	0

^aFor NJ analysis, the number of genes that yielded each tree with bootstrap support greater than 90% is given in parentheses.

^bFor Bayesian analysis, the number of genes that yielded each tree with posterior probability greater than 95% is given in parentheses.

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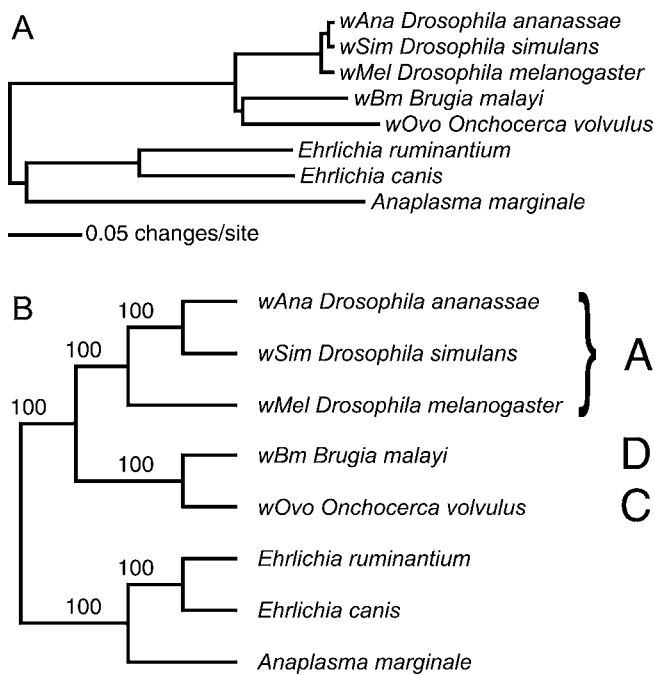


Figure 5. *Wolbachia* Relationships Inferred from 42 Protein-Coding Genes

(A) Phylogram of consensus tree with inferred distances based on the ultimate model parameters inferred in MrBayes, and rooted by the *Anaplasma* and *Ehrlichia* outgroups.

(B) Cladogram showing Bayesian support for each node.

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base). Neither of the *wOvo*-like segments in L13731 has a complete open reading frame because of the indel differences. Both of these *wOvo* genes have orthologues in *wMel* and *wBm*, but the region of *wOvo* OW4-C that is similar to L13731 is very divergent from the other *Wolbachia* genes (not shown). Alignment of the *wMel* and *wBm* orthologues of *wOvo* OWJ-2 (a predicted phosphomannomutase) to L13731 shows that the *O. volvulus* nuclear fragment is more similar to *wOvo* than it is to either of the other two *Wolbachia* (Figure 5A).

The nematodes from which Li and Donelson [33] prepared their genomic DNA derived from Mali. As the fragment was sequenced from a genomic DNA clone it was possible that it was a cloning artefact. This possibility was excluded by firstly amplifying the putative insertion from our independent source of *O. volvulus* specimens (from Ghana), and secondly by identifying an orthologous insertion in the genome of the related cattle parasite *O. ochengi*. We carried out PCR assays using primers designed to be able to amplify either from the putative insertion in the nuclear genome, or from the copy resident in the *wOvo* genome. We were able to amplify, and confirm by sequencing (Figure 6A), the presence of the *wOvo*-like segments upstream of the *O. volvulus* TATA box-binding protein gene (Figure 6B). *O. volvulus* is one of a group of onchocercid species endemic in Africa. It is known to be close phylogenetically to *O. ochengi*, a cattle parasite that has a range overlapping that of *O. volvulus*, with which it shares some vector species [34]. We surveyed the genomes of *O. ochengi* for *Wolbachia* from *O. ochengi* (*wOoc*) genes and the putative nuclear insertion and confirmed their presence. Sequencing of the putative insertion fragment (Figure 6A) revealed five

single base pair differences from *O. volvulus*. We were unable to confirm that the insertions were close to the *O. ochengi* TATA box-binding protein gene (unpublished data). By surveying the emerging genome sequence data for the filarial parasite *B. malayi*, we were able to identify a TATA box-binding protein gene, the orthologue of the *O. volvulus* gene, but did not find any significant sequence similarity to the *wOvo* gene fragments in the region upstream of this gene, and, indeed, did not identify any possible nuclear insertions of sequence similar to the five *wOvo* genome segments isolated in the *B. malayi* whole genome shotgun.

Discussion

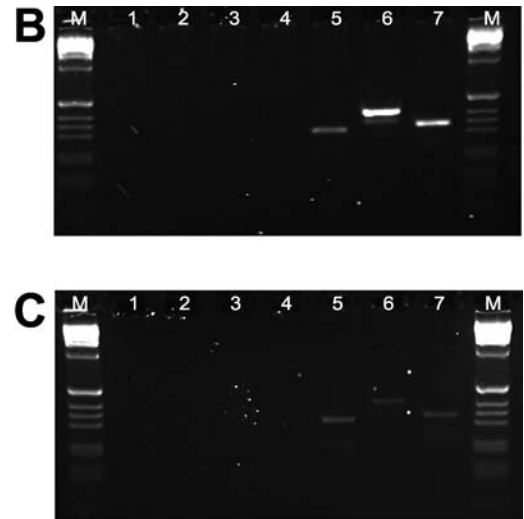
The Genome of *wOvo*

The sequenced segments yielded 70 kb of genome sequence for *wOvo*. Additional rounds of screening failed to yield further *wOvo* fragments, and construction of *Wolbachia*-enriched genomic libraries was unsuccessful. It would be very informative to complete the *wOvo* genome and we are continuing to investigate routes to this end.

Relationships of *Wolbachia* Revealed by Sequence Phylogenetics and Synteny

We analysed the sequence of the genes encoded in the five *wOvo* fragments for phylogenetic signal, as for these we could identify credible orthologues in outgroup taxa. For individual genes, the signal was mixed, but biased towards Tree 2 of Figure 1. However, under ML models, none of the individual genes gave strong support to either of Trees 1 or 2. We identified no particular functional annotation to separate those genes supporting Tree 1 from those supporting Tree 2 (Table 3). As combining genes can yield resolution of phylogenetic problems, by summing the minor signal present in each gene such that it was detectable above the background noise of homoplasy [24], we generated a concatenated alignment of 42 of the *wOvo* proteins and their orthologues. Analysis of this concatenated alignment using unpartitioned or partitioned (more realistic, given the variation in inferred rates between genes; Figure 4) models yielded robust support for Tree 1, i.e., [outgroups[[*wOvo*, *wBm*],[*wMel*,*wAna*,*wSim*]], equivalent to [outgroups[[C,D],[A]]] (Figure 5). Notably, the shortest inferred internal branch in the phylogeny was that linking the last common ancestor of all *Wolbachia* and the last common ancestor of the nematode (clade C and D) *Wolbachia*. The length of this branch compared to neighbouring ones in the phylogeny may explain the difficulty in robustly recovering a distinct phylogeny with more limited datasets. As genes from clade B *Wolbachia* are consistently very closely related to those from clade A rather than from clades C or D [1,35], we predict that inclusion of clade B in the analysis would yield a tree [outgroups[[C,D],[A,B]]].

Conserved gene arrangements (synteny) can be used to infer phylogenetic relationships between genomes. The *wOvo* fragments share some local synteny with both *wMel* and *wBm*. Where breakage of local synteny occurs, two features are apparent. Firstly, *wBm* and *wMel* are more similar to each other than either is to *wOvo*. Secondly, *wBm* is closer to *wOvo* than is *wMel*, as *wMel* has several unique rearrangements. Comparison to the outgroup genomes was uninformative because of the high levels of rearrangement that have taken place in *Wolbachia* genomes since they last shared a common



(B and C) PCR verification of the presence of the *Wolbachia*-like gene fragments in *O. volvulus* and its close relative *O. ochengi*. Ethidium-bromide-stained gels are shown with lanes M, DNA size markers; 1, 2, 3, and 4, single-primer controls for the primers TATA_Phos, TATA_OW4C, Phos, and OW4C, respectively (see Table 1 for primer sequences); 5, PCR product of wOvo phosphomannomutase (primers TATA_Phos and Phos); 6, PCR product of wOvo OW4-C (primers TATA_OW4C and OW4C); and 7, PCR product of the *Onchocerca* genomic insertion (primers TATA_Phos and TATA_OW4C). In (B) the target was *O. volvulus* genomic DNA, while in (C) the target was *O. ochengi* genomic DNA.

As a whole, the Rickettsiales have lifestyles that involve intracellular replication in a eukaryotic host cell, and the outgroups analysed here have parasitic or pathogenic lifestyles. The support for Tree 1 suggests that the ancestor of all extant *Wolbachia* was probably an intracellular pathogen or parasite. Our analyses suggest that this intracellular pathogen was then tamed by, or evolved beneficial symbiotic relationships with, its nematode hosts, but evolved towards specific reproductive parasitism in the arthropod-infecting clade A (and B) strains. A single transfer of an ancestral *Wolbachia* to

an onchocercid nematode host is most likely. The nematode *Wolbachia* have apparently coevolved with their hosts through strictly vertical descent, while the arthropod strains have undergone frequent (on an evolutionary timescale) horizontal transfers or host captures, while also maintaining themselves on a life-cycle timescale by vertical transmission. As arthropod *Wolbachia* are parasites, it is possible for individuals and populations to lose their infections. Importantly, it is also evident that nematodes can lose their *Wolbachia*, as *Wolbachia*-negative nematode species are nested within clades of infected taxa [16]. There is a correlation between the presence of WO phage in *Wolbachia* genomes [36] and the parasitic phenotype, and thus WO phage and/or genes transduced by WO phage may underpin parasitic manipulations [37]. There were no WO phage-like elements in the *wOvo* genome segments analysed.

Lateral Transfer of *Wolbachia* Genetic Material to the *O. volvulus* Nuclear Genome

Serendipitously, we identified two short fragments of *Wolbachia* genes in one of the few segments of the *O. volvulus* genome to have been sequenced. Transfer of *Wolbachia* genetic material into the host nuclear DNA has been noted previously, in the adzuki bean beetle, *Callosobruchus chinensis*, where a reasonably large segment of *Wolbachia* DNA has been inserted into the X chromosome [38]. The adzuki bean beetle insertion is not thought to be expressed.

The sequenced *O. volvulus* segment incorporates the gene for a TATA box-binding protein and a region 2 kb upstream. In this upstream region we detected two short segments that have significant pairwise identity to *wOvo* OW2-J and to *wOvo* OW4-C. We confirmed that the putative insertion was present in *O. volvulus* genomic DNA (and was not therefore a cloning artefact) by isolating it by specific PCR from an independent source of *O. volvulus*. Neither fragment is a complete gene, and both have been subject to mutational accumulation such that the open reading frames are no longer intact. The two genes do not lie beside each other in either the *wOvo* or *wBm* genomes. We suggest that an original insertion, perhaps of a relatively large portion of a *Wolbachia* genome, has been reduced by deletion, resulting in the close apposition of two fragmentary *Wolbachia* genes not found next door to each other in the bacterial chromosome. The insertional fragment is not unique to *O. volvulus*, as it is also present in the cattle onchocercid, *O. ochengi*. *O. ochengi* is very closely related to *O. volvulus*, and indeed *O. volvulus* in humans is thought to represent a recent host capture by, and vicariant speciation of, onchocercids of ungulates. No homologous insertion was detected in the partial genome sequence of *B. malayi*, but the orthologous TATA box-binding protein gene was identified. Examination of the region between the *B. malayi* TATA box-binding protein gene and the next gene upstream identified no sequences with significant similarity to the putative *Wolbachia* insertions (unpublished data). We also used PCR to screen for the insertion in the deer onchocercid *O. flexuosa*. *O. flexuosa* is interesting because it appears to lack *Wolbachia* entirely (as determined by PCR screens and electron microscopy) [39]. Identification of an insertional relic of *Wolbachia* would bolster suggestions that this species has lost its symbiont. However, we were unable to amplify any insertion fragments from *O. flexuosa* (unpublished data), leaving the question of symbiont loss unanswered.

Nuclear integration of fragments of other cytoplasmic genomes, such as the mitochondrial and chloroplast genomes, is relatively common, but no plausible integrants of *wBm* were detected in the near-complete *B. malayi* genome [26]. Whether acquisition of *Wolbachia* genes by the host plays any part in host evolution remains conjectural. Similarly, the *Wolbachia* could capture host genes, but none of the sequenced genomes contain genes with signatures of animal, rather than alphaproteobacterial, origin.

Materials and Methods

Selection of *wOvo* probes and identification of *wOvo* genomic clones. A series of probes were prepared from previously identified *wOvo* genes, including the 16S ribosomal RNA gene, *usp*, *ftsZ*, *hsp60*, and others identified in the *O. volvulus* EST (expressed sequence tag) programme [40,41] (Table 1). Probes were labelled with alpha32P dCTP by oligo-primed synthesis. *O. volvulus* libraries in lambda phage, gifts of John Donelson [33] and Steve Williams, were plated on bacterial lawns, and the lifts were prepared for Southern hybridisation using standard methods. Initial hybridisations used a mix of probes from several genes. After autoradiography, positive plaques were identified by gene-specific PCR, and purified by dilution and reprobing. Inserts were isolated by long-range PCR using lambda-vector primers, and end sequenced. End-probes were generated and used to reprobe plaque lifts. Primer sequences are given in Table 1.

Sequencing and annotation. Long-range PCR products were sequenced by standard shotgun methods at the Wellcome Trust Sanger Institute, and assembled using standard methods. The insert sequences were completed by a combination of directed sequencing of selected plasmid subclones, and primer walking. One clone insert proved to be a chimera of human and *Wolbachia* DNA; the human segment was identified by its sequence identity to human genomic sequence, and was removed from the analysis. Genes were identified and annotated in the *wOvo* genome segments using Artemis [42]. The Artemis comparative tool, ACT, was used to display and investigate synteny relationships with the *wBm* [26] and *wMel* [27] genomes. A putative *wOvo* HtrA serine protease (GenBank accession AAP79877) similar to OW4-E had been published previously [31]. To test if *wOvo* has more than one HtrA gene or if the difference was due to technical error, primers (see Table 1) were designed within the OW4-E 5' and 3' extragenic regions. Multiple PCR and sequencing reactions were performed according to standard procedures using *O. volvulus* genomic DNA. The sequences were aligned and a consensus sequence was obtained. To assess the possible function of the *wOvo*-specific gene, OW2-I, SignalP v3.0 [43] and pSortb v2.0 [44] were used to identify a possible signal peptide and a probable cellular location.

Phylogenetic analysis. For phylogenetic analysis, particularly since we desired to identify the root of the *Wolbachia* clade, it was essential to analyse alignments of orthologous sequences, and to exclude paralogues. Each protein-coding gene in *wOvo* was used to search (using BLAST [45]) a custom database of alphaproteobacterial proteins extracted from EMBL and GenBank to identify homologues. In addition, homologues were identified from the complete and partial genomes of *wBm*, *wMel*, *wAna*, *wSim* [46,47], *A. marginale* [48], *E. ruminantium* [49], and *E. canis*. For each *wOvo* protein, a multiple alignment was constructed using ClustalW [50] and subjected to NJ analysis in PHYLIP (using character difference) [51]. From the resulting phylograms we identified orthologous genes from the seven complete and partial genomes. Importantly, we excluded paralogues from genomes where an orthologue was absent. These paralogues were the best scoring match in the selected genome, but by phylogenetic analysis were clearly not orthologous to the *wOvo* query. The *wAna* and *wSim* genomes were assembled from whole genome shotgun reads "contaminating" those generated for the nuclear genome projects of their host species, and are incomplete. For *wAna*, we identified several genes that are present in one copy in other bacterial genomes but are duplicated (or partially duplicated) in the *wAna* assembly. We interpret these to be due to either misassemblies or the presence of two closely related *Wolbachia* genomes in *D. ananassae*. If one whole genome sequence shotgun survey includes DNA from two distinct *Wolbachia*, the genes we selected for subsequent analysis may be selected stochastically from two distinct genomes, but the close relationship implied by comparison of the "duplicated" segments in the assembly (>99% identity) means that they can effectively be considered a single taxon.

Ehrlichia and *Anaplasma* orthologues of two genes were not found, and in these cases we identified orthologues in *R. prowazekii* and *R. typhi* to use as outgroups.

For the 44 proteins with matches, and the 16S and 23S/5S ribosomal RNA genes, we realigned each *wOvo* sequence with its orthologues. The alignments are available as Protocol S1 online. The protein alignments were combined and subjected to phylogenetic analysis using NJ and Bayesian ML methods. NJ was carried out in PAUP 3.6 [52] with mean character distances. Bootstrap support was estimated for NJ trees by 1,000 resamplings. Bayesian analyses of protein-coding genes were carried out in MrBayes 3.1 [53] under the fixed rate JTT model of protein evolution with gamma rate variation approximated by four rate categories and a proportion of invariant sites. For RNA genes, DNA alignments were analysed under the HKY model with gamma rate variation (four categories) and a proportion of invariant sites. For each gene, two independent runs were executed for 1,000,000 generations, and sampled every 1,000 generations, with default prior and Markov chain parameters. After visual confirmation of stationarity, the first 10% of saved trees were discarded as burn in. The significance of the difference in support for the two credible alternative hypotheses was tested for each gene using a likelihood ratio test. *p*-Values were calculated using the SH test as implemented in Tree-Puzzle 5.1 (<http://www.tree-puzzle.de>) using accurate (slow) parameter estimation. Since, for many genes, one of the trees was the one selected by ML, this test is more appropriate than the Kishino-Hasegawa likelihood ratio test, which requires that trees be specified a priori. For protein-coding genes, amino acid alignments were analysed under the JTT model with gamma rate variation (four categories) and a proportion of invariant sites.

Rokas et al. [24] have shown that the use of large datasets, employing many genes with varying rates, is effective in recovering "correct" topologies when single-gene analyses fail to do so. Bayesian analyses of the concatenated alignment of 42 protein-coding genes was carried out under two models. In the first model, all genes shared a fixed rate JTT model of protein evolution with gamma rate variation approximated by four rate categories and a proportion of invariant sites. In the second model, the Poisson model was used, along with a rate multiplier that allowed each gene to evolve at a different rate. In addition, independent gamma rate parameters and proportions of invariant sites were estimated for each gene. For the concatenated analyses, two independent runs were executed for 2,000,000 generations and sampled every 100 generations, with default prior and Markov chain parameters. After visual confirmation of stationarity, the first 10% of saved trees were discarded as burn in. To test whether the second, more complex model gave a significantly better fit to the data, harmonic mean likelihoods from runs using different models were used to calculate Bayes factors.

PCR testing of lateral gene transfer. A potential lateral gene transfer event was detected through BLAST search of *O. volvulus* sequences in EMBL and GenBank using *wOvo* fragments and the *wBm*

genome as queries. The *Wolbachia* genes and their putative nuclear homologues were aligned using ClustalW (Figure 6). To prove the existence of the insertion in the *O. volvulus* genome, a series of oligonucleotide primers was designed (Table 1) that would be useful in PCR amplification of the insertion event in the nuclear genome and the genes resident in the *wOvo* chromosome. *O. volvulus* and *O. ochengi* DNA was isolated from nematodes from nodules using standard procedures. PCRs were carried out using ~100 ng of *O. volvulus* or *O. ochengi* DNA, and analysed on 1% agarose gels. Positive PCR fragments were isolated and sequenced to confirm their identity.

Supporting Information

Protocol S1. Multiple Sequence Alignments of *wOvo* Proteins and rRNAs Used in the Analysis of *Wolbachia* Relationships

The data are in NEXUS format.

Found at DOI: 10.1371/journal.ppat.0020094.sd001 (414 KB TXT).

Accession Numbers

Sequence data reported in this paper have been deposited in EMBL (<http://www.ebi.ac.uk/embl>), GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>), and DDBJ (<http://www.ddbj.nig.ac.jp>) with the accession numbers OW1 (CU062443), OW2 (CU062464), OW3 (CU062463), OW4 (CU062460), and OW5 (CU062461). The GenBank accession numbers for the *O. volvulus* (from Mali) TATA box-binding protein gene and a putative *wOvo* *HtrA* serine protease are L13731 and AAP79877, respectively. The GenBank accession number for the *E. canis* genome is CP000107.

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